REMARKS

Claim Objections

The Examiner objects to claims 22, 23 and 38-47 for utilizing acronyms. Accordingly, Applicants have amended the claims to recite the full term as suggested.

Claim Rejections 35 USC 112

The Examiner has rejected claims 38-41 and 50-52 under 35 USC § 112, first paragraph, as being non-enabling. Specifically, the Examiner contends that the specification does not teach the step of actively reducing pH. Applicants respectfully submit that one of skill in the art would be know of several means for reducing pH within an animal. For example, breath holding, inspiration of CO_2, exercise, instillation of Ringer's lactate, immersion in an acidic buffer and restriction of blood flow are all well understood mechanisms for altering pH within an animal or cell, *in vivo*. However, Applicants have amended claims 39 and 50 to clarify that actively reducing the pH is not required by the series of claims cited by the Examiner. Indeed, these methods should be understood to include the degradation of the encapsulator by the reduced pH within an endosome, which the Examiner identifies as occurring passively. Further, Applicants note that claim 38 as originally submitted does not recite a step of actively lowering pH. For these reasons, Applicants request that the Examiner withdraw the § 112, first paragraph, rejection of claims 38-41 and 50-52.

The Examiner also rejected claims 12, 33, 38-47 and 50-52 under 35 USC § 112, second paragraph. With respect to claims 12 and 33, the Examiner objects to the use of the term "derivatives." Applicants have amended the claims to replace the term "diketene acetal derivative" with "double ortho ester." The specification discloses that diketene acetal derivatives are examples of double ortho esters. With respect to claims 38-47, the Examiner states that the term "LOC" is not given a limiting definition in the specification. Applicants respectfully submit that "LOC" is defined in the specification, at page 3, in the "Summary of the Invention" as a "lipidic ortho ester conjugate." Applicants have also amended claims 38-47 and 50-52 to use the full term, rather than the acronym. Finally, the Examiner rejected claims 39 and 50-52 for failing to specify "what pH is to be reduced" and for a lack of antecedent basis. Applicants have amended claims 39 and 50-52 to address these problems. Specifically, the claims now

require "exposing the encapsulator to reduced pH." Thus, this clearly refers to the environment surrounding the encapsulator. Accordingly, Applicants request that the Examiner withdraw the § 112, second paragraph, rejection of claims 12, 33, 38-47 and 50-52.

Claim Rejections 35 USC 102

The Examiner rejected claims 1-8, 11 and 12 as being anticipated by Neville et al. The Examiner states that Neville discloses monoclonal antibodies linked to a toxin, linked in turn to a polyethylene glycol. The Examiner notes that the monoclonal antibodies and toxins disclosed by Neville may comprise a hydrophobic portion and that the disclosed linkages may be ortho esters.

Applicants have amended claim 1 to incorporate the limitations of claims 9 and 10, which are not disclosed by Neville. Applicants have also canceled claims 3, 4, 9 and 10 and changed the dependency of claims 7 and 8 to claim 19, which is addressed below. Therefore, Applicants request that the Examiner withdraw the § 102 rejection of claims pending claims 1, 2, 5, 11 and 12 over Neville.

The Examiner next rejected claims 1, 2, 4, 11-13, 19, 30-33, 38, 39 and 42 as being anticipated by Sparer. The Examiner states that Sparer discloses bioerodable poly(ortho esters) and beneficial agents, and specifically notes that a beneficial agent such as estradiol may be linked by an ortho ester to a polyethylene glycol. Applicants have amended claim 1 to emphasize that the ortho ester conjugate compositions of the invention are for incorporation into an encapsulator. Specifically, claim 1 now requires an "encapsulator selected from the group consisting of lipsomes, emulsions, micelles and lipidic bodies." Sparer does not disclose such encapsulators.

Applicants have amended claim 1 to incorporate the limitations of claims 9 and 10, which are not disclosed by Sparer

With respect to claim 19, the Examiner states that Sparer discloses an implant comprising crosslinked PEG and beneficial agent that "can be thought of as an encapsulator." Regarding claims 30-33, the Examiner states that the composition of Sparer appears to be substantially identical to the claimed composition. With respect to claims 38 and 39, the Examiner states Sparer's intended use for the composition is to deliver beneficial agents by implantation of the composition, followed by hydrolysis of the ortho esters. Applicants have amended independent claims 19, 30 and 38 to clarify that the encapsulators of the invention, as

defined in the specification, are liposomes, emulsions, micelles and lipidic bodies. Sparer, in contrast, does not disclose the claimed encapsulators.

Finally, regarding claim 42, the Examiner does not specifically state the portions of Sparer relied upon in reaching the conclusion of anticipation. Applicants respectfully request that the Examiner reconsider as claim 42 requires the step of mixing an encapsulator with a lipidic ortho ester conjugate. The Examiner has asserted that Sparer discloses that the "beneficial agent may be lipidic, e.g. estradiol" and that the crosslinked PEG and beneficial agent "can be thought of as an encapsulator." Applicants disagree with the conclusion that Sparer discloses an encapsulator, but even assuming the Sparer composition is an encapsulator, there is no disclosure of another composition that can be considered a lipidic ortho ester conjugate. Likewise, even if the Sparer composition is considered a lipidic ortho ester conjugate, there is no disclosure of a separate composition that functions as an encapsulator. Accordingly, Sparer cannot disclose the step of mixing a lipidic ortho ester conjugate with an encapsulator as required by claim 42.

For these reasons, Applicants request that the Examiner withdraw the § 102 rejection of pending claims 1, 2, 11-13, 19, 30-33, 38, 39 and 42 over Sparer.

Next, the Examiner rejected claims 1, 7-9, 19-21, 24-27, 30, 31, 34 and 35 under 35 USC § 102 as anticipated by Pease. The Examiner states that Pease discloses luminescent molecules, which may be hydrophilic, that are attached to liposomes by an ortho ester bond.

Applicants have amended claim 1 to incorporate the limitations of claims 3 and 4, which are not disclosed by Pease, canceled claim 9 and changed the dependency of claims 7 and 8 to claim 19, which is addressed below.

Applicants have amended independent claim 19 to clarify that hydrolysis of the ortho ester bond destabilizes the encapsulator. One of skill in the art would readily recognize that the liposomes disclosed by Pease does not become destabilized when the ortho ester bonds are hydrolyzed, nor does Pease disclose any other type of encapsulator. Pease discloses only the attachment of a fluorescent dye to the liposome via the ortho ester. Since the dye does not participate in the structure of the liposome, loss of the dye does not destabilize it. Further, Pease suggests that a detergent is preferably selected to release the dye from the liposome (see column 12, line 3). This implies that the release of the dye from the liposome does not destabilize the liposome of Pease. With respect to claims 30, 31, 34 and 35, these claims require that the

encapsulator degrade with acid hydrolysis. As discussed above, Pease does not disclose a degradeable encapsulator.

Therefore, Applicants request that the Examiner withdraw the § 102 rejection of pending claims 1, 7, 8, 19-21, 24-27, 30, 31, 34 and 35 over Pease.

The Examiner also rejected claims 50-52 under 35 USC § 102 as anticipated by Lishko. The Examiner asserts that this reference teaches the delivery of genetic material encapsulated in pH sensitive liposomes. Applicants have amended claim 50 to clarify that the encapsulator of the invention has an acid labile ortho ester bond. Lishko does not disclose such acid labile ortho ester bonds. Accordingly, Applicants request that the Examiner withdraw the § 102 rejection of claims 50-52 over Lishko.

The Examiner next rejected claims 1, 2, 4, 6-9, 15, 19, 20, 24, 28, 29, 30-34, 36, 38, 39, 42 and 50 under 35 USC § 102 as anticipated by Schacht. The Examiner states that Schacht discloses cationic polymer-based carrier vehicles bound by ortho ester linkers to hydrophilic polymers such as PEG. Applicants have amended claim 1 to incorporate the limitations of claims 9 and 10. Applicants respectfully submit that Schacht does not disclose the specific hydrophobic components now required by amended claim 1. Further, Applicants have canceled claims 4 and 9, and changed the dependency of claims 6-8 to claim 19, which is adressed below.

With respect to independent claims 19 and 30, Applicants have amended the claims to clarify that the encapsulator is degraded by hydrolysis of the ortho ester bonds and that the encapsulator comprises liposomes, emulsions, micelles, and lipidic bodies. Schacht does not disclose such encapsulators. With respect to claims 38, 42 and 50, Applicants respectfully submit that Schacht does not disclose the claimed lipidic orthoester conjugates as the reference does not teach lipids with ortho ester bonds. For these reasons, Applicants request that the Examiner withdraw the rejection of pending claims 1, 2, 6-8, 15, 19, 20, 24, 28, 29, 30-34, 36, 38, 39, 42 and 50 under 35 USC § 102 as anticipated by Schacht

Finally, the Examiner rejected claims 1, 7, 8, 15, 16, 19-21, 24, 25, 30, 31, 34, 35, 38, 39, 42 and 50 under 35 USC § 102 as anticipated by Nantz. The Examiner states that Nantz discloses ortho ester lipids having a hydrophilic portion linked by the ortho ester to a hydrophobic portion.

With respect to claim 1, Applicants have amended this claim to incorporate the limitations of claims 3 and 4. Since Nantz does not disclose these specific hydrophilic

components, Applicants respectfully submit that amended claim 1 and its dependents are not anticipated by Nantz. Please note that the Applicants have changed the dependency of claims 7 and 8 to claim 19, which is addressed below.

Regarding claims 19-21 (and thus, claims 7-8), 24, 25, 30, 31, 34, 35, 38, 39, 42 and 50, Applicants respectfully submit that the hydrophilic portion of Nantz is not directly dissociated from the hydrophobic portion by hydrolysis of the ortho ester bond. Referring to Fig. 2 of Nantz, the ortho ester hydrolysis step (Step I) occurs in going from compound 10 to the next compound (not numbered), but the two portions remain attached. The alcohol is not cleaved until a subsequent step (Step II) that is not an ortho ester hydrolysis, but rather an intramolecular transesterification, that produces lactone 12 and the amino alcohol 18. This chemistry is characteristic of all of the ortho esters disclosed by Nantz. Indeed, Nantz states "[i]t is this unique 2-step or tandem mechanism which facilitates liposome disassembly when compounds of Formula I are incorporated into liposomes." In sum, cleavage of the ortho ester in the Nantz patent does not directly separate the hydrophilic portion from the hydrophobic portion.

Applicants have amended independent claims 19, 30, 38, 42 and 50 to clarify that hydroloysis of the ortho ester directly detaches the hydrophilic portion from the hydrophobic portion. This feature is an inherent property of the ortho ester lipidic conjugates disclosed in the specification that would be readily apparent to one of skill in the art. For a further discussion of this inherent property, Applicants respectfully submit the reference Guo et al., Steric Stabilization of Fusogenic Liposomes by a low-pH Senitive PEG-Diortho Ester-Lipid Conjugate, *Bioconjugate Chem.* **2001**, 291-300 (Attached as Appendix A). Applicants are not trying to incorporate this material, but rather to demonstrate the inherent properties of the compounds disclosed in the specification as filed. As shown in Scheme 1, ortho esters such as those used in the specification release the attached alcohol in the first hydrolysis step. Hence, Applicants respectfully submit the specification inherently supports hydrolysis of the ortho ester directly detaching a hydrophilic portion from a hydrophobic portion of the lipidic compositions, as now required in independent claims 19, 30, 38, 42 and 50. As demonstrated above, the Nantz reference does not teach this feature.

Accordingly, Applicants request that the Examiner withdraw the rejection of pending claims 1, 7, 8, 15, 16, 19-21, 24, 25, 30, 31, 34, 35, 38, 39, 42 and 50 under 35 USC § 102 as anticipated by Nantz.

Claim Rejections 35 USC 103

The Examiner rejected claims 1-3 under 35 USC § 103 as being unpatentable over either Sparer or Schacht. The Examiner states that it would have been obvious to substitute methoxypolyethylene glycol for PEG. However, as discussed above, Applicants have amended claim 1 to incorporate the limitations of claims 8 and 9, and canceled claim 3. Since neither Sparer nor Schacht disclose the specific hyrdrophobic components now claimed, Applicants respectfully request that the Examiner withdraw the § 103 rejection of pending claims 1 and 2.

The Examiner next rejected claims 41, 48 and 49 under 35 USC § 103 as being unpatentable over Schacht in view of Lishko. However, as discussed above, Schacht does not teach the lipidic compositions comprising an ortho ester bond having the characteristics claimed. Lishko fails to disclose the use of ortho ester bonds at all. Accordingly, Applicants request that the Examiner withdraw the § 103 rejection of claims 41,48 and 49 over Schacht and Lishko

Finally, the Examiner rejected claims 48 and 49 under 35 USC § 103 as being unpatentable over Pease in view of Lishko. As discussed above, Pease does not suggest the use of degradeable encapsulators. Lishko fails to acid labile ortho ester bonds, and thus fails to compensate for the deficiencies of the primary reference. Applicants therefore request that the Examiner withdraw the § 103 rejection of claims 48 and 49 over Pease and Lishko.

Conclusion

Based on the above remarks and amendments, Applicants submit that the pending claims are patentable and request their early allowance. To expedite prosecution, the Examiner may contact the Applicants representative Nathan Koenig at (541) 806-2252.

Respectfully submitted,

CROSBY, HEAFEY, ROACH & MAY

Date: December 16, 2002 By: _____

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CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Box Amendment, Washington, D.C. 20231, on December 16, 2002, 2002.

Dated: December 16, 2002 By: _____
Rebecca M. Klits

Version of the Claims with Markings Showing Changes Made

1. (Amended) A composition comprising a hydrophilic portion and a hydrophobic portion joined by an ortho ester linker, wherein the ortho ester linker hydrolyzes at an increasing rate as the pH is reduced below 7 and wherein the hydrophilic portion is selected from the group consisting of methoxypolyethylene, polyethyleneglycol, hydroxylated dendrons, poly(methyloxazoline), poly(ethyloxazoline) and polyvinylpyrrolidone and wherein the hydrophobic group is selected from the group consisting of diacyl glycerols, distearoylglycerol, dipalmitoylglycerol, dimyristoyl glycerol, dioleoyl glycerol, tocopherol, cholesterol, coenzyme Q, and ceramide .

6. (Amended) The composition of claim [1] 19, wherein the hydrophilic portion comprises a targeting ligand.

7. (Amended) The composition of claim [1] 19, wherein the hydrophilic portion comprises a cationic group.

12. (Amended) The composition of claim 11, wherein the ortho ester linker comprises a [diketene acetal derivative] double ortho ester.

15. (Amended) The composition of claim [1] 19, wherein the ortho ester linker comprises a single ortho ester.

19. (Amended) A composition comprising an encapsulator selected from the group consisting of liposomes, emulsions, micelles and lipidic bodies, wherein the encapsulator comprises [the composition of claim 1] a hydrophilic portion and a hydrophobic portion capable of anchoring the composition to the encapsulator joined by an ortho ester linker, wherein the ortho ester linker hydrolyzes at an increasing rate as the pH is reduced below 7 and wherein hydrolysis of the ortho ester directly detaches the hydrophilic portion from the non-polymeric hydrophobic portion and destabilizes the encapsulator.

22. (Amended) The composition of claim 21, comprising DOPE/methoxypolyethylene glycol 2000-diortho ester-distearoyl glycerol conjugate (POD) in a ratio of about 97:3 to 85:15.

23. (Amended) The composition of claim 21, comprising DOPE/ dimethylethanolamine-ortho ester-cholesterol (DOC).

30. (Amended) An encapsulator for delivering a compound, comprising an amphipathic low pH sensitive lipidic composition comprising an ortho ester linker wherein the encapsulator exhibits degradation of less than 10% within 3 hours at a pH of 7.4 and degradation greater than 50% within 60 min at a pH of 5.0, wherein the encapsulator is selected from the group consisting of liposomes, emulsions, micelles and lipidic bodies, and wherein hydrolysis of the ortho ester linker directly detaches a hydrophilic portion of the lipidic composition from a hydrophobic portion of the lipidic composition to destabilize the encapsulator.

33. (Amended) The encapsulator of claim 32, wherein the ortho ester linker comprises a [diketene acetal derivative] double ortho ester.

38. (Amended) A method for delivering a drug to a cell comprising the steps of providing an encapsulator comprising a[n] lipidic ortho ester conjugate (LOC) and the drug, wherein the encapsulator is selected from the group consisting of liposomes, emulsions, micelles and lipidic bodies and wherein hydrolysis of an ortho ester linker directly detaches a hydrophilic portion of the lipidic ortho ester conjugate from a hydrophobic portion of the lipidic ortho ester conjugate to destabilize the encapsulator and administering the encapsulator.

39. (Amended) The method of claim 38, further comprising the steps of exposing the encapsulator to reduced [reducing] pH, degrading the encapsulator and releasing the drug.[Add support active lowering of pH]

42. (Amended) A method for incorporating a[n] lipidic ortho ester conjugates (LOC) into an encapsulator, the encapsulator comprising an ortho ester linker wherein hydrolysis of the

ortho ester linker directly detaches a hydrophilic portion of the lipidic ortho ester conjugate from a hydrophobic portion of the lipidic ortho ester conjugate to destabilize the encapsulator, comprising the step of mixing the encapsulator with the lipidic ortho ester conjugate (LOC).

43. (Amended) The method of claim 42, further comprising the steps of:

a) preparing a dry film of the lipidic ortho ester conjugate (LOC);

b) rehydrating the a[n] lipidic ortho ester conjugate (LOC) to form micelles; and

c) combining the micelles with an encapsulator suspension.

44. (Amended) The method of claim 42, wherein the encapsulator comprises a cationic lipoplex further comprising the steps of preparing a cationic lipoplex and coating the lipoplex with the lipidic ortho ester conjugate (LOC).

45. (Amended) The method of claim 42 further comprising the steps of:

a) preparing a dry film of the lipidic ortho ester conjugates (LOC);

b) preparing an encapsulator suspension; and

c) combining the encapsulator suspension with the dry film.

46. (Amended) The method of claim 42, further comprising the steps of :

a) preparing the lipidic ortho ester conjugate (LOC) in a non-aqueous, water miscible solvent

b) preparing an encapsulator suspension; and

c) combining the encapsulator suspension with the lipidic ortho ester conjugate (LOC) in the water miscible solvent.

48. (Amended) A method for storing an encapsulator for delivering a compound, comprising the steps of:

a) providing an encapsulator comprising an amphipathic low pH sensitive lipidic compound comprising an ortho ester linker wherein the encapsulator exhibits degradation of less than 10% within 3 hours at a pH of 7.4 and degradation greater than 50% within 60 min at a pH of 5.0, and wherein hydrolysis of the ortho ester linker directly detaches a hydrophilic portion of

the lipidic ortho ester conjugate from a hydrophobic portion of the lipidic ortho ester conjugate to destabilize the encapsulator; and

b) lyophilizing the encapsulator.

50. (Amended) A method for gene transfer comprising the steps of:

a) providing an encapsulator comprising an amphipathic low pH sensitive lipidic composition having an acid labile ortho ester bond and a polynucleotide, wherein hydrolysis of the ortho ester linker directly detaches a hydrophilic portion of the lipidic composition from a hydrophobic portion of the lipidic composition to destabilize the encapsulator;

b) administering the encapsulator to an animal;

c) exposing the encapsulator to reduced [reducing the] pH to degrade the encapsulator; and

d) releasing the polynucleotide.

Steric Stabilization of Fusogenic Liposomes by a Low-pH Sensitive PEG–Diortho Ester–Lipid Conjugate

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We describe the synthesis and characterization of a pH-sensitive poly(ethylene glycol)-diortho ester-distearoyl glycerol conjugate (POD). POD was prepared by a one-step synthesis, and its acid sensitivity characterized by TLC. The conjugate was found to be stable at neutral pH for greater than 3 h but degraded completely within 1 h at pH 5. Liposomes composed of 10% of POD and 90% of a fusogenic lipid, dioleoyl phosphatidylethanolamine (DOPE) were readily prepared and remained stable for up to 12 h in neutral buffer as shown by photon correlation spectrometry and a liposome contents leakage assay. However, when POD/DOPE liposomes were incubated in acidic pH as mild as 5.5, they aggregated and released most of their contents within 30 min. The kinetics of content release from POD/DOPE liposomes consisted of two phases, a lag phase, and a burst phase. The lag phase is inversely correlated with pH and the logarithm of the length of lag phase showed a linear relationship with the buffer pH. When the POD/DOPE liposomes were incubated in 75% of fetal bovine serum at 37 °C, they remained as stable as traditional PEG-grafted liposomes for 12 h but released 84% of the encapsulated ANTS in the following 4 h. Upon intravenous administration into mice, liposomes composed of 10% POD and 90% DOPE were cleared from circulation by a one-compartment kinetics with a half-life of about 200 min. POD is an example for the design of a novel category of pH sensitive lipids composed of a headgroup, an acid-labile diortho ester linker and a hydrophobic tail. The uniquely fast degradation kinetics of POD at pH 5–6 and its ability to stabilize liposomes in serum make the conjugate suitable for applications for triggered drug release systems targeted to mildly acidic bio-environments such as endosomes, solid tumors, and inflammatory tissues.

INTRODUCTION

Diacyl chain lipids represent an important class of amphipathic molecules that contain both a polar headgroup and a large hydrophobic moiety. In aqueous phase, such lipids self-assemble into colloidal particles such as liposomes, micelles and hexagonal phase. Liposomes are widely used as models of membrane bilayers and carriers of a variety of diagnostics and medicines (*1*). Examples of their use in drug delivery include PEG stabilized liposomes that carry cytotoxic drugs to leaky tumor tissues (*2*) and cationic liposomes that improve gene transfection (*3*).

Ideally, a targeted drug delivery system, including liposomes, should remain stable until it reaches the target site to minimize the premature loss of its payload (*2, 4*). Upon accumulation at the target site, drug release needs to be at a high enough level for an effective therapeutic response. Since the decrease of pH is implicated in many physiological and pathological progressions such as endosome processing (*5*), tumor growth (*6*), inflammation (*7*), and myocardial ischemia (*8*), it has been extensively exploited to trigger the release of drugs from liposomal delivery systems in the past 20 years (*9–11*). Most of the reported pH-sensitive liposomes are based on the neutralization of excess negative charges on their surface upon protonation, which reduces the hydrodynamic diameter of lipid headgroups and triggers the change of lipid bilayers to hexagonal phases (*10*).

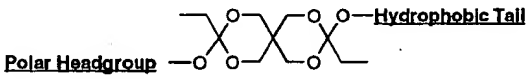

Figure 1. General structure of the diortho ester conjugates.

However, at neutral pH, these excess negative charges induce undesired interactions with serum proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation (*12, 13*). Efforts to circumvent this difficulty and provide a nonionic pH-sensitive lipid have employed a neutral monosaccharide as the headgroup, which is attached to long hydrophobic chains via an acetal moiety (*14*). However, these acetal analogues hydrolyzed relatively slowly at pHs 5 to 7 as found in physiological environment and need a pH less than 4 for rapid degradation. Recently, Thompson and co-workers have reported a number of acid- and light-sensitive lipids containing vinyl ether moieties (*15–17*). Although vinyl ethers represent a promising approach, their rate of hydrolysis at pH 5 is not optimal for rapid drug release. To develop a versatile strategy to prepare biocompatible liposomes, which are sensitive to small pH decrease, we propose a novel class of lipids (Figure 1) that possess an acid-labile diortho ester linker between the hydrophilic headgroup and the hydrophobic tail. In this case, a variety of headgroups and tails could be conjugated together to render pH-sensitive lipids of different properties.

Ortho esters are relatively stable under basic and neutral conditions but hydrolyze quickly at acidic pH due to a stabilized dialkoxy carbocation intermediate (Scheme

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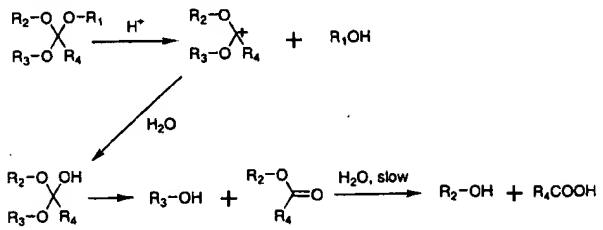
Scheme 1. Mechanism of Ortho Ester Hydrolysis



1) (*18–20*). Heller and co-workers (*21*) have prepared a series of polymers based on the diortho ester moiety and exploited their pH-sensitive properties to develop sustained drug release systems. As an example of the proposed diortho ester lipidic conjugates, we report in this paper the design, preparation, and characterization of a novel lipid (1, POD, Scheme 2)[1] that consists of a PEG headgroup, a diortho ester linker, and a distearoyl glycerol hydrophobic tail. We show that POD can be used to prepare long circulating and acid-triggerable liposomes in combination with DOPE, a lipid that by itself does not form stable liposomes, but hexagonal phases at neutral pH.

EXPERIMENTAL PROCEDURES

General Techniques. The diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane was received as a generous gift from Dr. Jorge Heller at Advanced Polymer Systems (Redwood City, CA). Monomethyl ether of PEG (MW 2000) was purchased from Shearwater Polymers, Inc (Huntsville, AL). Distearoyl glycerol was purchased from Genzyme (Cambridge, MA). Triethylamine was purchased from Aldrich and redistilled under Ar before use. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt (POPG), dioleoylphosphatidylethanolamine (DOPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma. 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and *p*-Xylenebis(pyridinium) bromide (DPX) were from Molecular Probes, Inc. (Junction City, OR). MilliQ water, which had a pH of approximately 8 when freshly prepared, was used to prepare all the aqueous buffers. All other chemical reagents and solvents were purchased from Aldrich or Fisher. Ratios of components in chromatography solvent systems are in volume unless stated otherwise. Ratios of lipid components in liposomes are in mole units.

^{1}H NMR spectra were recorded on an Oxford AS 400. Electrospray mass spectra (ESIMS) were recorded on a Sciex (PE, Foster City, CA) at the Mass Spectrometry Facilities, University of California at San Francisco. Fourier-transformed infrared spectra were measured with a Nicolet Impact 400. The FT-IR samples were applied as a $CHCl_3$ solution onto the surface of a NaCl crystal, dried under Ar, and subsequently analyzed. Elemental analysis was carried out by Microanalytical Lab at College of Chemistry, University of California at Berkeley.

3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(methoxypoly(ethylene glycol)2000-1-yl)-2,4,8,10-tetraoxaspiro[5,5]undecane (POD, 1). One gram of PEG (2000) monomethyl ether (0.5 mmol) and 312.5 mg of distearoyl glycerol (0.5 mmol) were dissolved in 5 mL of anhydrous THF under Ar. A heat gun was used to melt 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane and 100 μL (110 mg, 0.5 mmol, d = 1.1) of the melted compound was taken by a dry syringe and injected into the THF solution. One drop (~40 μL) of 0.6 mg/mL *p*-toluenesulfonic acid in anhydrous THF was added and the reaction mixture was stirred at 40 °C under Ar for 2 h. The reaction was stopped by adding 200 μL of triethylamine followed by quenching the reaction into 30-fold volume excess of 1% volume of triethylamine in methanol. Four grams of silica gel were added to the solution and the mixture was evaporated under reduced pressure. The residue was poured onto a silica gel column (45 g) equilibrated with the eluting solvent system (triethylamine/chloroform = 1/50). The column was eluted and fractions corresponding to the product were pooled, evaporated, and dried in high vacuum to give 275 mg (20%) purified product. TLC = R_f 0.4 in $CHCl_3$/MeOH/30% w/w NH_4OH (100:15:1); FTIR 2910 cm^{-1} (CH_2 and CH_3), 2850 cm^{-1} (CH_2 and CH_3), 1743 cm^{-1} (ester C=O), 1109 cm^{-1} (PEG and ortho ester C–O); ^{1}H NMR (400 MHz, $CDCl_3$, chemical shifts relative to TMS signal) δ 5.22 (1H, m, glycerol methine), 3.7–4.2 (4H, m, glycerol methylene), 3.0–3.7 (~170 H, m, OCH_2 and OCH_3), 2.2–2.4 (4H, m, CH_2COO), 1.65–1.76 (4H, m, CH_2CH_2COO), 1.54–1.65 (4H, m, CH_2CH_3 on spiro rings), 1.04–1.36 (56H, m, $CH_3(CH_2)_{14}CH_2CH_2COO$), 0.82–0.97 (12H, m, CH_2CH_3); ESIMS, calcd for $[M + Na]^+$ with 36–48 CH_2CH_2O units from PEG: $C_{123}H_{240}O_{46}Na$ 2476.6, $C_{125}H_{244}O_{47}Na$ 2520.7, $C_{127}H_{248}O_{48}Na$ 2564.7, $C_{129}H_{252}O_{49}Na$ 2608.7, $C_{131}H_{256}O_{50}Na$ 2652.7, $C_{133}H_{260}O_{51}Na$ 2696.8, $C_{135}H_{264}O_{52}Na$ 2740.8, $C_{137}H_{268}O_{53}Na$ 2784.8, $C_{139}H_{272}O_{54}Na$ 2828.8, $C_{141}H_{276}O_{55}Na$ 2872.9, $C_{143}H_{280}O_{56}Na$ 2916.9, $C_{145}H_{284}O_{57}Na$ 2960.9, $C_{147}H_{288}O_{58}Na$ 3004.9, found 2478.3 (33%), 2521.1 (47%), 2565.3 (62%), 2609.3 (77%), 2654.2 (94%), 2697.3 (100%), 2741.4 (98%), 2785.3 (93%), 2830.4 (85%), 2874.4 (60%), 2917.5 (46%), 2962.4 (37%), 3006.4 (26%). Anal. calcd for $C_{135}H_{264}O_{52}$, C 59.62, H 9.78; $HC_{137}H_{268}O_{53}$, C 59.54, H 9.77; $C_{139}H_{272}O_{54}$, C 59.46, H 9.76; $C_{141}H_{276}O_{55}$, C 59.39, H 9.76; $C_{143}H_{280}O_{56}$, C 59.31, H 9.75; $C_{145}H_{284}O_{57}$, C 59.24, H 9.74; $C_{147}H_{288}O_{58}$, C 59.17, H 9.73; found C 59.16, H 9.57, N<0.2.

Thin Layer Chromatography (TLC) Analysis of POD Degradation in Buffers of Different pH at 37 °C. Conical polystyrene microcentrifuge tubes (0.5 mL) containing 20 μL of 100 mM sodium phosphate buffer of different pH were prewarmed at 37 °C for 30 min and silica gel TLC plates (Whatman from Whatman Ltd, Maidstone, Kent, England) were preequilibrated with the solvent system ($CHCl_3$/MeOH/ NH_4OH = 100:15:1) by running the plates without samples followed by brief air-drying. Eight milligrams of POD was dissolved into 100 μL of water by vortexing and 20 μL of the solution was added into each of the prewarmed tubes and mixed thoroughly. The tubes were incubated at 37 °C and 10 μL of the solutions were aliquoted at different time points and mixed with 10 μL of 10% concentrated NH_4OH (30% w/w) in acetone to stop the hydrolysis. Five microliters

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; BPE, *p*-hydroxybenzamidine phosphatidylethanolamine; CHEMS, cholesteryl hemisuccinate; Chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylenebis(pyridinium) bromide; DSG, 1,2-distearoyl glycerol; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000; HEPES, (hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; mPEG, PEG(2000) monomethyl ether; PEG, poly(ethylene glycol); POD, poly(ethylene glycol) 2000-diortho ester-distearoyl glycerol conjugate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt; SD, standard deviation; TLC, thin-layer chromatography.

of the mixture was spotted onto preequilibrated TLC plates. Three microliters of distearoyl glycerol, monomethyl ether of PEG2000 and POD in 10 mg/mL $CHCl_3$ solution were also spotted as standards. TLC plates were developed, heated at 150 °C for 5 min and stained in I_2 chamber for 1 h to observe the locations of POD and its degradation products.

Liposome Preparation for Aggregation and Leakage Assays. Reversed-phase vesicles (REV) were prepared as described previously (*22*) in 50 mM ANTS, 50 mM DPX, and 5 mM HEPES at pH 8.5. The vesicles were extruded five times through a 0.2-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) through a hand held extrusion device (Avestin, Ottawa, Ontario, Canada). A Sephadex G-75 column was used to separate vesicles from unencapsulated material with an elution buffer composed of 5 mM HEPES and 145 mM NaCl, pH 8.5. All freshly prepared liposomes had mean diameters ranging from 180 to 200 nm (cumulant results) and a polydispersity index of less than 0.2 as measured by a Malvern Zeta1000 Dynamic Light Scattering Instrument using the PCS 1.32a software. The encapsulated volume was 0.25−0.3 μL/μmol total lipid. Lipid concentrations were determined based on lipid phosphorus by a modification of the Bartlett method (*23*).

Liposome Aggregation Assay. Conical polystyrene microcentrifuge tubes (0.5 mL) containing 300 μL of buffers of various pH (50 mM NaOAc/HOAc and 100 mM NaCl for pH <6; 50 mM NaH_2PO_4/Na_2HPO_4 and 100 mM NaCl for pH 6 and above) were incubated at 37 °C for 30 min. The liposome preparation was warmed to room temperature and an aliquot (100 μL) was added to each tube and mixed thoroughly. The tubes were incubated at 37 °C for different time periods and 30−40 μL of the mixtures were aliquoted and transferred into disposable polystyrene fluorimeter cuvettes containing 1.5 mL of 100 mM $NaHCO_3$/50 mM NaCl buffer at pH 9. The samples were mixed by gently inverting the cuvettes 2−3 times.

Photon correlation spectrometry of the samples was then measured with a Malvern apparatus (Zeta 1000) to determine the size distribution of the liposomes. Typically, three measurements of 2−5 min were made for each sample, using the automatic algorithm mode for data analysis. The average of the three cumulant results of each sample that agreed to within 10% is reported in the paper.

ANTS/DPX Leakage Studies. The ANTS/DPX assay (*24*) was used to monitor leakage of ANTS from liposomes. Fluorescence measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source. Excitation was at 370 nm (1.25 mm slit). The 90° emission signal at 550 nm (5 mm, 5 mm slits) resulting from the dequenching of ANTS released out of liposomes was observed through a Corning 3-68 nm cutoff filter (>530 nm). The raw fluorescent data were converted into ASC data files and processed mathematically by Microsoft Excel. The residual fluorescence of the liposomes at the starting time (t_0) of leakage experiments, F_0, was set as 0% release. At the end of each leakage experiment the liposomes were lysed with the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) and the maximal fluorescence thus obtained, F_{100}, was taken as 100% release. The leakage of ANTS at a particular time point was then determined by the formula: percentage of leakage = $(F_t - F_0)/(F_{100} - F_0) \times 100$, where F_t corresponds to the fluorescence intensity observed at the time point.

In leakage experiments of POD/DOPE liposomes in aqueous buffers of different pHs, the assays were started by injecting small volumes (5−10 μL, using a Hamilton syringe) of concentrated liposome suspensions into a magnetically stirred quartz cuvette containing 2 mL of the buffer (50 mM NaOAc/HOAc and 100 mM NaCl, pH <6; 50 mM NaH_2PO_4/ Na_2HPO_4 and 100 mM NaCl, pH 6 and above) at 37 °C. The starting time, t_0 was set 5−10 s after the injection when the fluorescence signal first became stable.

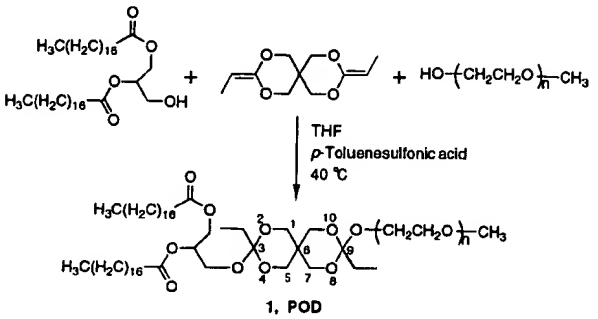
In serum stability assays, aliquots of fetal bovine serum (180 μL in each tube) in 0.6 mL tubes were prewarmed at 37 °C for 30 min. An aliquot of stock liposome solution was diluted to 2 mM of total lipid by phosphate buffered saline (50 mM NaH_2PO_4/Na_2HPO_4 and 100 mM NaCl, pH 7.4), which had been prewarmed at 37 °C for 30 min. An aliquot of the diluted liposome solution (60 μL) was then mixed with 180 μL of prewarmed fetal bovine serum to give a 75% final serum concentration. At different time points, a 20 μL aliquot was added into a polystyrene fluorimeter cuvette, containing 2 mL of HEPES-buffered saline (5 mM HEPES and 145 mM NaCl, pH 8.5) at room temperature. The cuvette was covered with Parafilm and inverted gently for 3−4 times. The fluorescence thus measured subtracted by the fluorescence of the buffer before the addition of the sample was taken as F_t. The F_t of an aliquot extracted and measured immediately after mixing a liposome sample with fetal bovine serum was taken as F_0. At the end of each assay an aliquot of the liposome-serum mixture was pipetted into 2 mL of HEPES buffered saline (5 mM HEPES and 145 mM NaCl buffer, pH 8.5), followed by adding the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$). The fluorescence measured after adding the detergent minus the fluorescence of the buffer was taken as F_{100}. Each data point of the leakage assay in fetal bovine serum represents the average and standard deviation of three independent samples.

Animals. Blood clearance, distribution and excretion studies were conducted on 4-week-old female ICR mice (about 25 g in weight) purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times with a sodium pentobarbital overdose.

Preparation of ^{125}I-Labeled Liposomes for Animal Studies. *p*-Hydroxybenzamidine phosphatidylethanolamine (BPE) was synthesized and radiolabeled with $Na^{125}I$ as previously described (*25*) and the purity assessed by thin-layer chromatography. The labeled lipid contained less than 0.5% of free ^{125}I. The labeled lipid, in chloroform solution, was added to POD/DOPE (1/9) or DSPE-PEG/DOPE (1/9) mixture in chloroform to yield 8.3 × 10^{11} dpm/mol of total lipid. Chloroform was evaporated under reduced pressure and the resultant lipid film was placed under high vacuum for 2 h to remove the remaining solvent. The dried lipid film was hydrated with HEPES buffered saline (10 mM HEPES and 145 mM NaCl, pH 7.4) over 20 min by intermittent agitation on a vortex mixer and the suspension extruded three times through a 0.1 μm polycarbonate membrane. The diameter of the extruded liposomes, as measured by photon correlation spectrometry, was 140−160 nm. Each mouse received a tail-vein injection of 150 μL of the extruded liposomes containing 0.9 μmol total lipid.

Blood Clearance Assay. At various time points following administration of ^{125}I-labeled liposomes, ani-

Scheme 2. Synthesis of PEG–Diortho Ester–Distearoyl Glycerol Conjugate (POD, 1)



1, POD

mals were anesthetized with inhalation of isofluorane. Approximately 50 μL of blood was collected with a glass pasteur pipet from the orbital sinus vein, weighed and its γ-activity measured in Beckman Gamma 8000 (Fullerton, CA). The γ-activity in whole blood and hence, the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood/gram of animal weight. The average and standard deviation of samples collected from three animals were presented.

Tissue Distribution Assay. At various time points following administration of ^{125}I labeled liposomes, animals were anesthetized with an intraperitoneal (i.p.) injection of 100 μL of anesthetics cocktail (44 mg/kg ketamine, 2.5 mg/kg xylazine, and 0.75 mg/kg acepromazine). Approximately 1 mL of blood was removed *via* intracardiac puncture. One milliliter of phosphate-buffered saline (containing 0.2 g/L KH_2PO_4, 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/L KCl, and 8.0 g/L NaCl) was perfused through the right cardiac ventricle. The heart, lungs, liver, spleen, kidneys, stomach, intestines, head, mid section, and tail were collected and weighed, and the radioactivity counted in the Beckman Gamma 8000. The γ-activity in whole blood and hence the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood/gram of animal weight. The average and standard deviation of samples from three animals were reported.

RESULTS

Design and Synthesis of PEG–Diortho Ester–Distearoyl Glycerol Conjugate (POD, 1). To devise a lipid that degrades specifically in a low-pH environment but is inert under neutral physiological conditions, we chose PEG as the headgroup since it is one of the most stable synthetic polymers in vivo and liposomes coated by PEG have a prolonged circulation period (*26*). The monomethyl ether of PEG-2000 was used for synthesis to preclude cross-linking or polymerization. The 3,9-diethyl-2,4,8,10-tetraoxaspiro[5,5]undecane moiety was chosen as the diortho ester linker, based on previous research by Heller and co-workers (*27*), who showed its pH sensitivity and biocompatibility in polymeric drug delivery systems. Distearoyl glycerol, with two saturated hydrocarbon side chains, is attached to the diortho ester linker as the hydrophobic tail to anchor the conjugate into lipid bilayers (*28, 29*). If the conjugate is incorporated into liposomes of unsaturated phosphatidylethanolamine, distearoyl glycerol would be regenerated in the bilayers upon hydrolysis, and would favor the formation of hexagonal phases due to its conical structure (*30*).

POD was prepared by a one-step synthesis as illustrated in Scheme 2. The monomethyl ether of PEG2000 and distearoyl glycerol were dissolved in dry THF under atmospheric Ar. The solid diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane was melted with a heat gun under atmospheric Ar and added with a syringe to the THF solution to avoid contact with air. The reaction was then initiated by adding a trace amount of *p*-toluenesulfonic acid dissolved in dry THF. The mixture was stirred at 40 °C under Ar for 2 h and the reaction was stopped by addition of triethylamine. Silica gel chromatography with a solvent system containing 2% triethylamine as a stabilizer afforded the purified conjugate (20% synthetic yield) that appears homogeneous on TLC. Although the yield of the reaction was modest, we chose this synthetic route since the method is quick and the reagents are relatively inexpensive.

It should be noted that POD is actually a mixture of conjugates with the mPEG headgroup comprising of a narrow distribution of molecular weights around 2000, due to the polydisperse nature of the commercially available PEG2000 monomethyl ether. The electrospray mass spectrometry showed a distribution of peaks centered on 2697 Da, corresponding to monosodiumated molecular cations with different numbers of ethylene glycol units. The heterogeneity of mPEG as well as the stereochemistry of the conjugate also resulted in NMR peaks of POD appearing as multiplets, a feature which has been previously reported in the literature (*31*).

Acid Sensitivity of POD. To quickly estimate the sensitivity of the conjugate toward mildly acidic conditions relevant to biological scenarios, we employed a simple TLC assay to monitor the degradation of POD after it was incubated for different time periods at 37 °C in buffers of pH ranging from 4 to 7.4. As shown in Figure 2, within 1 h of incubation at 37 °C, POD was completely degraded in buffers of pH 4 and 5 as indicated by the disappearance of the compound spot at $R_f \approx 0.4$. However, in the buffer of neutral pH 7.4, most of the conjugate remained intact over 3 h. In the buffer of pH 6, POD degraded with an approximate half-life of 2.5 h. As expected from the acid-catalyzed hydrolysis illustrated in the Introduction, the degradation of POD yielded both more hydrophilic ($R_f \approx 0.3$) and more hydrophobic ($R_f \approx 0.8$) materials, presumably derivatives of mPEG and distearoyl glycerol, respectively. The hydrophilic degradation products gave more intensely stained spots than the hydrophobic derivatives, because mPEG derivatives are much more sensitive to iodine staining then are the saturated acyl chains. Similar results were obtained when the TLC plates were stained with Molybdate reagent, which clearly showed the hydrophobic degradation products (Data not shown).

Liposome Formation. Encouraged by the TLC results on POD degradation, we proceeded to study whether POD incorporated into a high percentage of DOPE, a fusogenic lipid, can stabilize a lamellar structure in neutral and alkaline solutions, and yet induces destabilizing phase changes upon cleavage of its mPEG headgroup by hydrolysis at low pH. ANTS and DPX (*24*) were encapsulated into liposomes by reverse phase evaporation (*22*) and their leakage from the vesicles were used to monitor pH triggered release of liposomal contents. Liposomes of defined size were prepared by extruding five times through polycarbonate membranes of 200 nm pore diameter. Unencapsulated ANTS/DPX was then removed by size-exclusion chromatography. Liposomes composed of 10% POD and 90% DOPE were readily prepared and remained stable for up to 2 weeks in an alkaline HBS buffer (5 mM HEPES, 145 mM NaCl, pH 8.5) at 4 °C with no significant increase in particle size or in residual ANTS fluorescence.

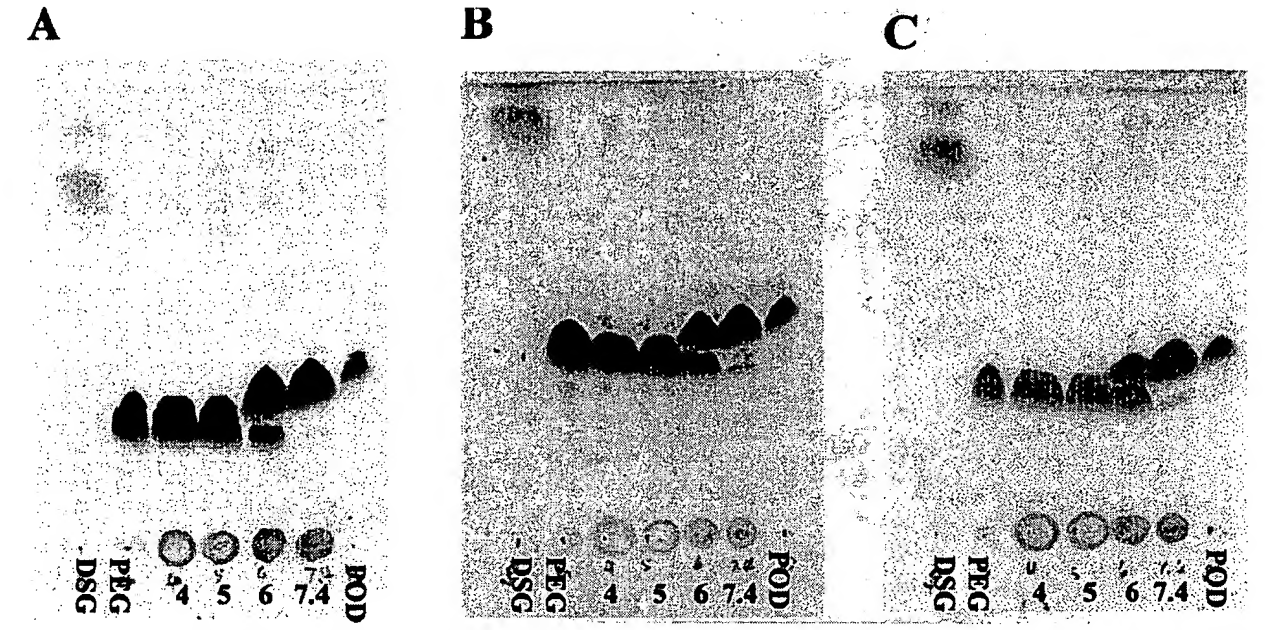


Figure 2. TLC monitored degradation of POD. Lipids were incubated for the period of 1 h (A), 2 h (B), and 3 h (C) in Na_2HPO_4 buffers of the pH indicated under the application origin. POD, mPEG, and DSG in $CHCl_3$ solution were spotted alongside as standards.

Acid-Triggered Liposome Aggregation. When pH sensitive liposomes composed of phosphatidylethanolamine (PE) are subjected to low pH, the PE lipids undergo a contact-induced aggregation and a phase transition from the lamellar to the hexagonal phase (*24*). PEG lipids at a high enough concentration on the liposome surface can shield the PE lipids of one liposome from contacting those of another liposome, thus preventing aggregation and membrane-destabilizing phase changes. Thus one can follow the loss of the PEG coating from the liposome surface as a result of POD hydrolysis by monitoring the aggregation of POD/DOPE liposomes at different pHs with photon correlation spectroscopy (PCS). However, as indicated by the TLC assays, the kinetics of the aggregation, particularly at pH below 5.5, may be too fast to be monitored by PCS, which takes about 10–20 min for a triplicate measurement. To circumvent this problem, we elected to "freeze" the aggregation process by quenching aliquots of samples in excess buffer of pH 9. The quenching stopped the acid-catalyzed POD hydrolysis and prevented the liposomes/lipid particles from contacting each other by inducing negative charges to their surfaces via deprotonation of the amine in DOPE. We found "base-freezing" to be a valid method to prevent size increases in the aggregation products. To determine if quenching of the reaction to pH 9 resulted in dispersion of aggregates into smaller particles, we took aliquots of liposomes incubated at pH 6 and diluted them in the same pH 6 buffer. The particle size of the liposomes was then immediately determined by a single PCS measurement of 2 min. The size data thus obtained were not significantly different from the diameters measured after "base-freezing". Furthermore, the samples quenched at pH 9 gave the same PCS readings after being left overnight at room temperature. Thus, the base quenching yielded a reproducible value that reflected the particle size distribution at the quench time. Therefore, the light scattering of the aliquots quenched at pH 9 was recorded and the particle diameter from the cumulant results was plotted against incubation time in Figure 3.

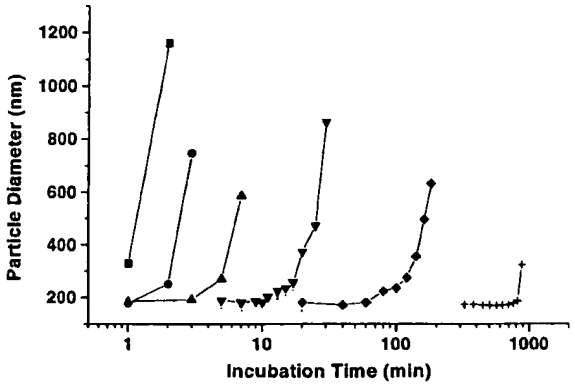


Figure 3. Aggregation of POD/DOPE (1/9) liposomes in buffers of different pH. (■) pH 4; (●) pH 4.5; (▲) pH 5; (▼) pH 5.5; (♦) pH 6.2; (+) pH 7.5.

The aggregation process showed a pH-dependent lag phase followed by a rapid increase in diameter (Figure 3). At pH 7.5, the POD/DOPE liposomes were relatively stable and the aggregation did not occur after more than 10 h of incubation at 37 °C. In buffers of lower pH, the lag phase was much shorter. At pH 6.2, the liposome size started to increase after 1 h and when the pH was decreased to 5, extensive aggregation was observed within 10 min. Thus, the kinetics of the liposome aggregation correlated with that of pH-dependent POD degradation shown by the TLC studies. These results demonstrate the ability of POD to protect the DOPE-containing liposomes from aggregating and converting into hexagonal phases until the mPEG headgroups are removed from the surface of the liposomes at low pH.

Acid-Triggered Content Leakage. Since POD was designed for potential applications in liposomal drug delivery, we used POD/DOPE liposomes containing ANTS/DPX as a model system to examine if the hydrolysis of POD can trigger the release of the contents from DOPE liposomes in response to a low pH environment. The ANTS/DPX method is often used to study pH sensitive liposomal leakage since the fluorescence of ANTS is

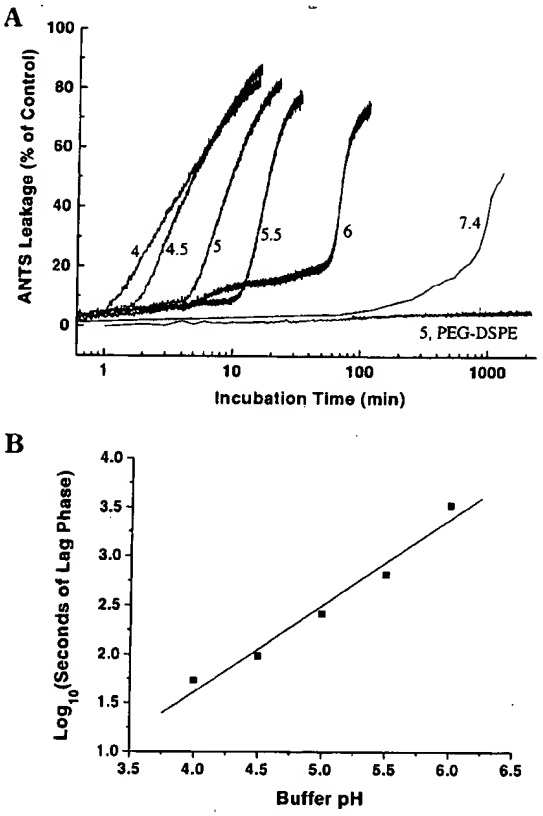


Figure 4. Acid-triggered content release from POD/DOPE (1/9) liposomes. (A) ANTS leakage in buffers of different pH. The pH is indicated adjacent to the corresponding trace. DSPE-PEG/DOPE (1/9) liposomes were incubated at pH 5 as a control. (B) Length of lag phase in buffers of different pH. The logarithm of the length of lag phase in seconds is regressed versus the buffer pH to give $\log_{10}$ (seconds of lag phase) $= -1.92 + 0.883 \times$ pH, $r = 0.984$.

virtually unchanged from pH 4 to 8 (*24*). When both compounds remain encapsulated, the fluorescence of ANTS is quenched by DPX (*24*). Upon leakage from the liposomes, ANTS is no longer quenched by DPX and gives an increase of fluorescent signal at 550 nm (excitation wavelength = 370 nm). As shown in Figure 4A, the release of ANTS at different pH also occurs in two distinct phases, a lag phase, where a small portion of ANTS slowly leaks out of the liposomes, followed by a burst phase, when most of the ANTS is quickly released. The burst phase correlates well with the aggregation of the POD/DOPE liposomes in Figure 3 and the collapse of the lamellar phase. As the liposomes are subjected to more acidic environments, the lag phase shortens. At pH 7.4, the lag phase lasts approximately 12 h; at pH 6, the lag phase is reduced to 60 min; at pH 5, the lag phase is less than 5 min. As a control, ANTS/DPX was encapsulated into liposomes composed of 90% DOPE and 10% of a pH *insensitive* PEG lipid, DSPE-PEG. Less than 5% of the encapsulated ANTS was released over 12 h when the vesicles were treated in the same way as the POD/DOPE liposomes even at the lowest pH. This confirms the role of acid-catalyzed hydrolysis of the PEG–diortho ester–DSG conjugate in the leakage of the POD/DOPE liposomes.

In Figure 4B, we plot the logarithm of the length of lag phase in Figure 4A against buffer pH from 4 to 6 and observe a clear linear relationship ($r = 0.984$). The lag time at pH 7.4 was not plotted in the graph since the

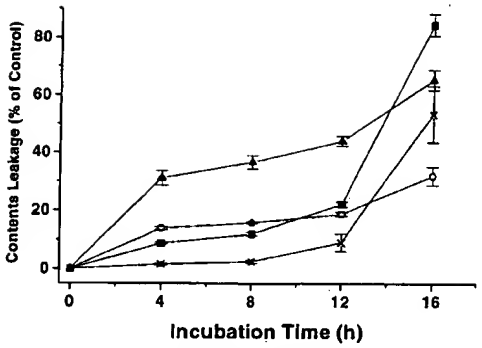


Figure 5. Contents leakage from liposomes of various compositions in 75% fetal bovine serum. ANTS leakage was monitored after the liposomes were incubated in 75% fetal bovine serum at 37 °C for different time periods. (■) POD/DOPE (1/9); (○) DSPE-PEG/DOPE (1/9); (▲) CHEMS/DOPE (3/7); (×) POPG/POPC/Chol (1/9/8).

transition is too slow to allow a precise determination. The slope of the regressed line is 0.883. This linear relationship demonstrates that the buffer pH plays a pivotal role on the kinetics of liposomal leakage. This phenomenon is discussed in detail in the discussion section, where we propose a mathematical model to account for the data.

Stability of POD Containing Liposomes in Serum. To test the idea that POD should help stabilize liposomes in circulation, we carried out an in vitro stability test in fetal bovine serum. POD/DOPE (1/9) liposomes containing ANTS/DPX were incubated with 75% fetal bovine serum at 37 °C and the release of ANTS was measured every 4 h for 16 h (Figure 5). For comparison, ANTS/DPX was also encapsulated into liposomes composed of DSPE-PEG/DOPE (1/9), CHEMS/DOPE (3/7), POPG/POPC/Chol (1/9/8), and the ANTS leakage was monitored in the same manner.

POD/DOPE liposomes were found to be relatively stable over 12 h, when less than 25% of the ANTS leaked out. However, after 16 h of incubation, 84% of the ANTS was released, indicating a transition from the lag phase to the burst phase. The POD/DOPE liposomes were as stable as the pH-insensitive, PEG-stabilized DSPE-PEG/DOPE liposomes for the first 12 h, but released ANTS much faster for the next 4 h, indicating a liposome destabilization process specifically mediated by POD hydrolysis. In the first 12 h of incubation, the POD/DOPE liposomes released 2–3-fold less ANTS than CHEMS/DOPE liposomes, whose excess negative charges from CHEMS tend to induce more interactions with serum components (*12*). The stable liposome formulation composed of POPG/POPC/Chol released less than 10% of the contents after 12 h of incubation with serum (*32*). Thus, POD is able to stabilize liposomes in fetal bovine serum as effectively as traditional PEG-derived lipids for up to 12 h, which is consistent with our findings on ANTS leakage of these liposomes in HEPES buffered saline. (Figure 4).

Stability of Liposomes in Blood Circulation. The steric-stabilizing effect of POD on liposomes in vivo was evaluated by tail-vein injection of radiolabeled liposomes composed of 10% POD and 90% of DOPE into female ICR mice and measuring their rate of elimination from the blood (Figure 6). For comparison, we also administered radiolabeled liposomes composed of 10% DSPE-PEG, a pH-insensitive lipid–PEG conjugate, and 90% DOPE. Liposomes composed of pure DOPE are not stable enough at pH 7.4 to allow their injection into animals. For both

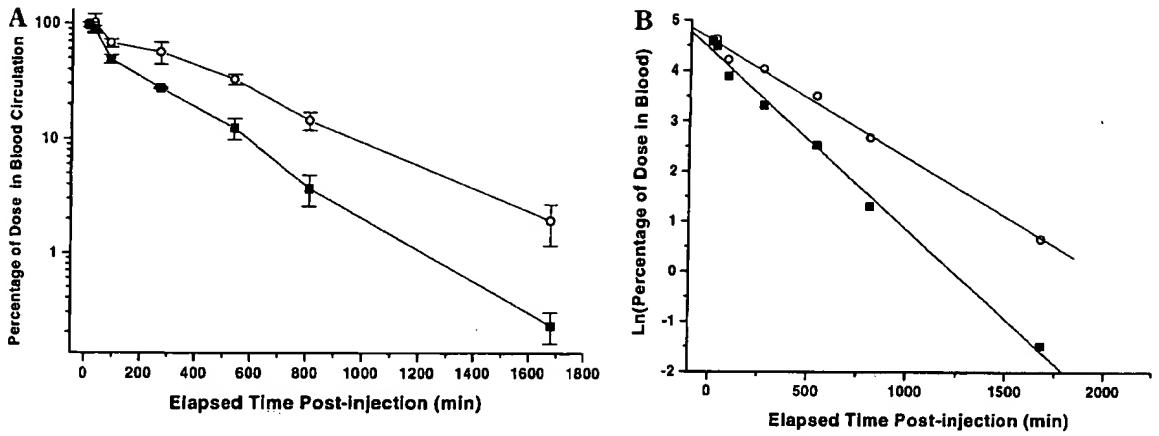


Figure 6. Clearance of [^{125}I]BPE POD/DOPE (1/9) liposomes (■) and [^{125}I]BPE DSPE-PEG/DOPE (1/9) liposomes (○) from circulation. (A) The percentage of the injected dose remaining in blood (n = 3, mean ± SD) is plotted against the elapsed time postinjection. (B) The natural logarithm of the percentage of dose remaining in blood is plotted against elapsed time postinjection and regressed to obtain the liposomal half-life. For POD/DOPE (1/9) liposomes, ln (percentage of dose in blood) = 4.41 − 0.003 58 × (min), r = 0.997, $T_{1/2}$ = ln 2/0.00358 = 193.6 min. For DSPE-PEG/DOPE (1/9) liposomes, ln (percentage of dose in blood) = 4.62 − 0.002 35 × (min), r = 0.997, $T_{1/2}$ = ln 2/0.00235 = 295.0 min.

POD/DOPE and DSPE-PEG/DOPE liposome formulations, the percentage of injected dose remaining in circulation showed a single log-linear decay with a correlation coefficient constant greater than 0.995, indicating a one-compartment clearance kinetics. The blood clearance profile of the POD/DOPE liposomes is similar to the pH-insensitive, sterically stabilized DSPE-PEG/DOPE liposomes than to conventional liposomes, which usually exhibit a two-compartment elimination kinetics (*33*). The half-life of the POD/DOPE liposome and the DSPE-PEG/DOPE liposome is about 194 and 295 min, respectively. This reasonably close correspondence of the elimination rate between the two formulations is strong evidence that the mPEG headgroup in the pH-sensitive ortho ester conjugate provides a steric-stabilizing effect on the fusogenic DOPE liposomes in circulation.

Distribution and Excretion of Liposomes. To investigate the distribution of radiolabeled POD/DOPE liposomes in more detail, we measured the radioactivity associated with different organs of mice at 270 min, 810 min and 28 h following liposome injection. In previous studies with the ^{125}I-labeled BPE (*25*), it is known that this radioactive lipid label is metabolized only after being endocytosed together with the liposomes. The urine and the feces were also collected at the 28-h time point and their radioactivity measured to determine the routes of ^{125}I excretion. At 270 min postinjection (Figure 7A), about 20% of POD/DOPE liposomes and 45% of DSPE-PEG/DOPE liposomes remained in circulation. For both formulations, the liver and intestine were the two major organs of radioactivity disposition.

At later time points (810 min and 28 h postinjection), most of the radioactivity of both formulations was excreted from the animals (Figure 8); the liver and intestine were the major organs for the disposition of the remaining radioactivity in the body (Figure 7B, 7C). Twenty-8 h after injection, the residual radioactivity from POD/DOPE liposomes was distributed preferentially to the head (Figure 7C), probably due to an accumulation of the iodine in the thyroid followed by a slow clearance of ^{125}I from this organ. For both formulations at 28 h postinjection, about 70% of radioactivity was excreted into urine and about 20% was found in feces (Figure 9). Overall, POD/DOPE liposomes were eliminated from circulation about 50% faster than DSPE-PEG/DOPE liposomes but the patterns of distribution and excretion of the two formulations were very similar.

DISCUSSION

The novel pH-sensitive ortho ester lipid derivative introduced here is a useful addition to the other reported pH-sensitive linkages for modifying liposome surfaces. The synthesis is simple and versatile. Although we illustrate the process with monomethyl ether of PEG, the chemistry could be used to attach various other headgroups to diacyl/steryl hydrophobic groups. For instance, anionic groups, cationic groups and even targeting ligands can be attached to a lipid anchor to create a triggerable headgroup for liposome formulations, micelles and other particulates. Modified glycerols, dendrons (*34*), or poly(vinylpyrrolidone) (*35*) could also be conjugated to lipids using this chemistry.

Regarding to the synthesis of POD, it should be noted that the acid-catalyzed conjugation does not produce POD as the unique product. Our preliminary TLC observations suggested that the side-products of the reaction include the conjugate of two mPEG or two distearoyl glycerol molecules on both ends of the diortho ester linker. The presence of side reactions, along with the loss of POD during its chromatography purification, may account for the moderate yield of the synthesis.

POD showed a remarkably quick degradation even at a mildly acidic pH of 5−6. Such sensitivity to acidic pH is significantly higher than that of acetals, vinyl ethers, and polyortho esters. We attribute the higher sensitivity of POD to the following two factors. First, the dialkoxy carbocation intermediate of ortho ester hydrolysis has four lone pairs of electrons over which to distribute the positive charge from the carbon, and hence is much more stable (Scheme 1) than the monoalkoxy carbocation intermediate, stablized by two lone pairs of electrons, in the case of acetals and vinyl ethers. Second, the PEG headgroup of POD is more hydrophilic than the functional groups of the reported polyortho esters, allowing better hydration and faster proton transfer to the diortho ester linkage (*36*). When POD was incorporated into liposomes composed of a high percentage of DOPE, POD hydrolysis at pH 5−6 triggered the extensive aggregation and leakage of the liposomes in 10−100 minutes.

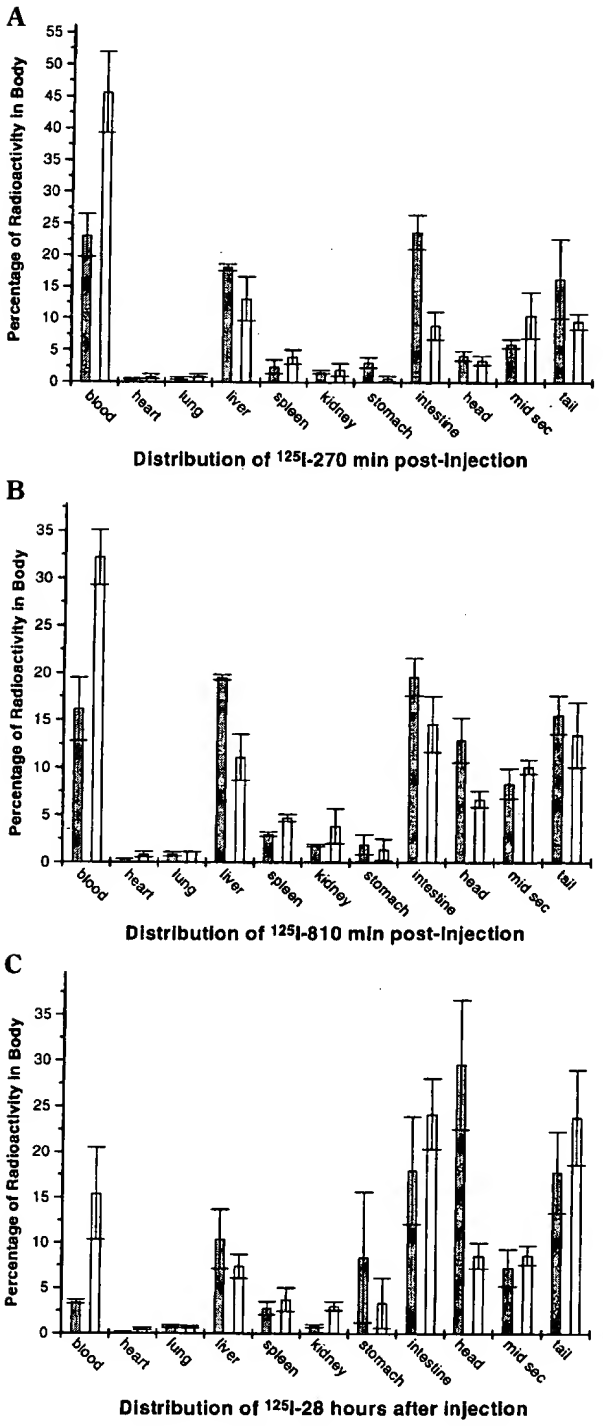


Figure 7. Distribution of [^{125}I]radioactivity of POD/DOPE (1/9) (solid bars) and DSPE-PEG/DOPE (1/9) (open bars) liposomes in female ICR mice at 270 min (A), 810 min (B), and 28 h (C) postinjection. (n = 3, mean ± SD).

We believe that the rapid hydrolysis of POD at mildly acidic pH is a very useful property for drug/gene delivery systems because the decrease of pH at potential therapeutic sites may be only one pH unit or less. For example, the transit through the endosomes in cells occurs in about 10 to 30 min with pH in the range of 5−6 before the endosomal contents are delivered into the lysosome (*37*, *38*). Therefore, it is important for pH sensitive liposomes

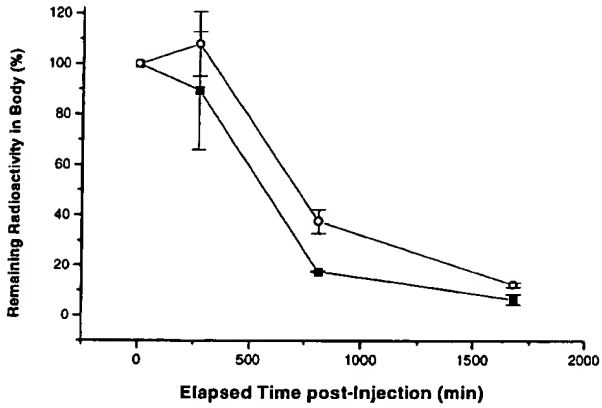


Figure 8. Excretion kinetics of [^{125}I]radioactivity of POD/DOPE (1/9) (■) and DSPE-PEG/DOPE (1/9) (○) liposomes.

to respond quickly to the initial drop in pH and release their contents prior to trafficking into the lysosomal compartment. The pH-dependent release profile of POD/DOPE liposomes may also be important for triggered release at inflammatory tissues (*7*, *40*) and solid tumors (*6*, *39*), where the pH is only 0.5−1 unit more acidic than that of the circulation.

The lag phase of ANTS leakage (Figure 4) corresponds quite closely to the lag time needed for liposome aggregation as measured by the increase of particle diameter (Figure 3). On the basis of these observations, we propose the following model of acid-catalyzed destabilization of POD/DOPE liposomes. Once the liposomes are subjected to an aqueous phase of mildly acidic or neutral pH, the mPEG headgroups are continuously cleaved off the liposome surface as shown in Scheme 1. However, the lipids remain in the lamellar phase until the amount of mPEG on the liposome surface decreases to a critical level, at which point the bilayers of two vesicles can come in contact. The bilayer contact is what triggers the aggregation of liposomes, the transition to the hexagonal phase and the release of liposome contents (*24*). The model is consistent with the known properties of PEG coated liposomes (*41*) and the requirement of bilayer contact to trigger the lamellar-hexagonal transition in PE liposomes (*24*). Assuming complete hydration and unaltered local pH at the liposome surface, the following equation holds:

$$dA/dt = -kA[H^+] \quad (1)$$

where A is the number of mPEG groups on the surface of a liposome of certain size, $[H^+]$ is the proton concentration of the aqueous medium, and k is the rate constant for ortho ester hydrolysis. In a buffer of constant pH, the equation can be integrated to give

$$A = A_0 \exp(-k[H^+]t) \quad (2)$$

where A_0 is A at an arbitrary starting point of POD hydrolysis and t is the incubation time from the starting point. At the transition point between the lag phase and the burst phase of liposomal leakage, we have

$$A_c = A_0 \exp(-k[H^+]t_c) \quad (3)$$

where A_c is the critical amount of mPEG on the surface of the liposomes necessary to stabilize the lamellar structure and t_c is the length of the lag phase. If the same liposome preparation is incubated in buffers of different pH, both A_c and A_0 should be constant and we can write

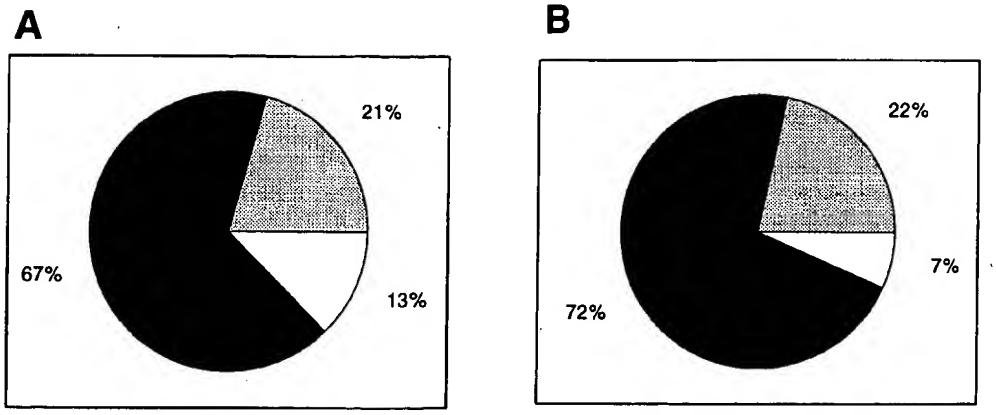


Figure 9. Mass balance of injected radiolabeled liposomes. [^{125}I]Radioactivity of POD/DOPE (1/9) (A) and DSPE-PEG/DOPE (1/9) (B) liposomes from all parts of body (white), urine (black), and feces (gray) were measured 28 h postinjection and plotted in percentage of the total radioactivity recovered. The percentage of injected dose recovered is 110% for POD/DOPE liposomes and 93% for DSPE-PEG liposomes.

the following equation:

$$[H^+]t_c = C \quad (4)$$

where C is a composite constant composed of A_c, A_o, and k. After taking the logarithm of eq 4 followed by rearrangement, the length of the lag time and the pH of the buffer would have the following relationship:

$$\log t_c = pH + C \quad (5)$$

where C is a constant.

Equation 5 fits closely to the observed leakage data in Figure 4B. The linear relationship between the logarithm of observed lag time and buffer pH, as well as the slope of the regressed line in Figure 4B (0.883 versus 1 in eq 5), support this mechanistic model of acid triggered phase changes of POD/DOPE liposomes. Further biophysical studies are required to confirm this model but the simple model is consistent with previous studies on lamellar-hexagonal transition as well as the data presented in Figure 2, 3, 4, and 5.

When incubated in 75% fetal bovine serum, POD was able to stabilize POD/DOPE (1/9) liposomes for up to 12 h, a finding consistent with the lag time for ANTS leakage of POD/DOPE liposomes in HEPES buffered saline at the same pH. Thus the stability characteristics vis-à-vis drug retention of this two component formulation are compatible for targeted delivery of encapsulated molecules given the observed intravenous elimination rate ($T_{1/2}$ = 194 min) of the formulation. Based on the distribution and the excretion data, we propose that both formulations were cleared mainly by liver from circulation, after which the [^{125}I]BPE labels were degraded and excreted into urine and bile.

The half-life of the POD/DOPE formulation in circulation is similar to other formulations that consist primarily of DOPE and that are stabilized by PEG (*42*, *43*). It is not surprising that such formulations have a shorter serum half-life than the traditional sterically stabilized formulations that consist of saturated phosphatidylcholine/Chol/PEG-DSPE (*44*). Such composition yields an inherently more rigid bilayer that is capable of resisting the penetration of serum components that might contribute to a more rapid elimination of a liposome even when it has a steric coat. Thus, we suspect that further improvements of the serum half-life and drug release characteristics can be achieved by modification of the lipid composition such as by the inclusion of cholesterol in the formulation (*32*).

CONCLUSION

In conclusion, a PEG−diortho ester−distearoyl glycerol conjugate (POD) was designed and prepared by a one-step synthesis. POD was characterized for its pH sensitivity and ability to stabilize liposomes in serum and in blood circulation. POD was found to be highly sensitive to acidic conditions but relatively stable at neutral pH. Liposomes composed of 10% POD and 90% DOPE aggregated and released their contents at a mildly acidic pH similar to that found in an endosome. The destabilization of POD/DOPE liposomes consists of two phases, a lag phase where encapsulated contents slowly leaked from the vesicles, and a burst phase that was associated with liposome aggregation and rapid release of contents. Liposomes composed of 10% POD and 90% DOPE remained stable in 75% fetal bovine serum at 37 °C for 12 h and had a half-life of about 200 min in blood circulation of female ICR mice. The fast kinetics of acid-catalyzed POD hydrolysis and its ability to stabilize liposomes in serum and in blood circulation may provide important advantages for the lipid to be used for drug and gene delivery.

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